FACTORIAL SCREENING OF FERULIC ACID PRODUCTION FROM FIBER PRESSED OIL PALM FROND USING MIXED CULTURE

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ABSTRACT

Ferulic acid (FA) belongs to the family of phenolic acid and is abundantly found in vegetables and fruits. It is one of the organic compound antioxidant and is extensively used in many industries due to its properties such as anti-inflammatory, antimicrobial and anti-carcinogenic. FPOPF is a renewable substrate and abundantly available in Malaysia therefore it was used as the substrate for the production of FA. Mixed culture was used instead of single culture and co-culture because of its ability to facilitate biological process under non-sterile condition. The fractional factorial screening experiments were performed for parameters of inital pH value, incubation temperature, fermentation time, agitation rate and substrate-to-inoculum ratio. The purpose of the fractional factorial screening was to determine the effect of those independent parameters on the production of FA from FPOPF using mixed culture. There were a total of 16 runs of experiments performed and the most contributing parameters to FA production from FPOPF were determined using Design Expert software. The ANOVA indicated that the model was significant with a coefficient of determination value of 0.8978. Temperature and pH value provided two of the highest contribution to the process. There were two factor interactions discovered throughout the process, interaction between factor A, temperature and factor B, pH value; and interaction between factor A, temperature and factor C, agitation.

ABSTRAK

Asid ferulik (FA) tergolong di dalam kumpulan asid fenolik yang banyak terdapat di dalam sayur-sayuran dan buah-buahan. Ia adalah salah satu sebatian antioksida organik dan mempunyai penggunaan yang meluas di dalam pelbagai industri kerana ciri-ciri yang terdapat padanya seperti anti-keradangan, agen antimikrob dan agen anti karsinogenik. Objektif kajian ini adalah untuk menjalankan *fractional factorial design* ke atas penghasilan FA daripada sabut pelepah kelapa sawit yang dikisar (FPOPF) menggunakan kultur campuran. FPOPF dipilih sebagai substrat kerana ianya mudah diperbaharui dan banyak terdapat di dalam Malaysia. Kultur campuran dipilih menggantikan kultur tunggal dan kultur bersama kerana keupayaan kultur campuran memudahkan proses biologi di bawah keadaan tidak steril. *Fractional factorial design* telah dijalankan ke atas parameter eksperimen yang berikut; nilai pH awal, suhu pengeraman, masa fermentasi, kadar pengadukan dan nisbah substrat kepada inoculum. Tujuan *fractional factorial design* ini adalah untuk mengenal pasti kesan setiap parameter eksperimen ke atas penghasilan FA daripada FPOPF menggunakan kultur campuran. Jumlah keseluruhan eksprimen ini dijalankan adalah 16 kali dan faktor yang paling banyak memberi kesan terhadap penghasilan FA daripada FPOPF dikenalpasti melalui perisian Design Expert. Keputusan ANOVA menunjukkan bahawa model eksperimen ini signifikan dengan nilai pekali penentuan 0.8978. Suhu pengeraman dan nilai pH merupakan faktor yang paling banyak memberikan kesan kepada proses ini. Sebanyak dua interaksi antara faktor telah dikenalpasti sepanjang proses ini, interaksi antara faktor A, suhu dan faktor B, nilai pH; dan interaksi antara faktor A, suhu dan faktor C, kadar pergolakan.

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LIST OF ABBREVIATIONS

BHA	Butylhydroxyanisole
BHT	Butylhydroxytoluene
FA	Ferulic acid
FE	Fermented extract
FPOPF	Fiber pressed oil palm frond
HPLC	High performance liquid chromatography
OPEFB	Oil palm empty fruit branch
OPF	Oil palm frond
OPT	Oil palm trunk
UFE	Unfermented extract

1 INTRODUCTION

1.1 Motivation and statement of problem

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) belongs to the phenolic acid family and known to be abundantly comes from vegetables and fruits (Mancuso *et al.,* 2013). There is a great interest in producing FA since it is known to have a wide range of application which is in pharmaceutical, cosmetics, and food industry. It has been reported that FA can act as antioxidant which may offer beneficial effects against cancer, cardiovascular diseases, diabetes and Alzheimer's disease (Zhao *et al*., 2008).

FA can be produced via chemical synthesis and bioconversion. By chemical synthesis, FA can be produced by condensation reaction of vanillin with malonic acid catalyzed by piperidine (Ou *et al*., 2007). Meanwhile via bioconversion, FA can be produced by conversion of lignocellulosic biomass such as grass, leaf fruit, vine and white grape stems as feedstock (Salgado *et al*., 2012).

Nowadays, biomass utilization for value-added products has been widely discussed. Among the many types of biomass, lignocellulosic biomass has become the most favourable substrates to be used in bioconversion for the value-added product as it is renewable and abundantly obtainable. (Zahari *et al.,* 2014). There were several extensive researches that have been completed on conversion of lignocellulosic biomass to other products including FA, ethanol and butanol. Bioconversion of lignocellulosic biomass into FA was achieved with the aid of some bacteria, fungi and yeast in either aerobic or anaerobic pathway (Hasyierah *et al*., 2008).

Oil palm frond (OPF) is one example of lignocellulosic biomass that is easily obtainable in Malaysia, as they are generated daily at oil palm plantation. Fiber pressed OPF (FPOPF) is the by-product from OPF processing. FPOPF also contains sufficient amount of carbohydrate that is useful to be used as fermentation feedstock (Zahari *et al.,* 2014). Other than reducing cost of production and abundantly available, using lignocellulosic biomass in bioconversion of value-added products such as FA could reduce the net greenhouse emission and contribute in conserving the environment.

Therefore this research intends to determine the effect of five independent variables upon the production of FA from FPOPF using mixed culture using the approach of fractional factorial screening.

1.2 Objective

The following are the objective of this research:

- To screen five independent variables for the production of ferulic acid from fiber pressed oil palm frond using mixed culture.

1.3 Scope of this research

The scope of this study is to do factorial screening for FA production from FPOPF using mixed culture. The factorial screening includes experimental parameters:

- pH value (pH 5 to pH 9)
- Incubation temperature (26°C to 40°C)
- Fermentation time (1 to 3 days)
- Agitation rate (150 rpm agitation and without agitation)
- Substrate-to-inoculum ratio (2%-10%)

1.4 Main contribution of this work

The main contribution of this research is that it was utilizing the potential of FPOPF, lignocellulosic biomass from palm oil industry that is generated and discarded daily in Malaysia. By doing so, this research has therefore helps in conserving the environment. In addition, this research was also able to performed FA extraction, which offers accessible business fortuity, and provides supplementary environmental and economic encouragement for industries as it is used in ingredients of many drugs, functional foods and nutraceuticals (Kumar & Pruthi, 2014)

1.5 Organisation of this thesis

Chapter 2 provides a description of FA application in industry and a review on production of FA. There are two methods to produce FA which are by chemical synthesis; such as the condensation reaction of vanillin with malonoic acid, and by bioprocess; for example enzymatic methods to release FA from natural sources. Other

than that, this chapter also describes the parameters that affect production of FA via bioprocess. In addition, this chapter also provides a review on FPOPF and mixed culture as fermentation feedstock.

Chapter 3 gives the review of materials and chemical reagents used in this research which are FPOPF, FA, sodium hydroxide (NaOH) and acetonitrile for high performance liquid chromatography (HPLC) analysis. Besides that, this chapter also describes the methods of FA fermentation which involves substrate preparation, inoculum preparation, experimental setup and HPLC analysis for the detection of FA production.

2 LITERATURE REVIEW

2.1 Overview

This research presents the fractional factorial screening on the production of FA from FPOPF using mixed culture. The fractional factorial screening was done on five independent experimental parameters which are initial pH value, incubation temperature, fermentation time, agitation rate and substrate-to-inoculum ratio. This chapter describes the two methods of FA production which are by chemical synthesis and by bioprocess. This chapter also describes the application of FA in various industries including medical and cosmetics industry. In addition, it also gives review on the use of FPOPF and mixed culture as fermentation feedstock.

2.2 Introduction

Ferulic acid was first time discovered when an Austrian Hlasiwetz Barth, isolated 4-hydroxy-3-methoxycinnamic acid from a plant named *Ferula foetida* for its structure determination, and hence it is named based on that botanical plant (Kumar & Pruthi., 2014). In 1925, it is structurally confirmed that FA are presence in two isomeric forms; *cis* and *trans* conformation (Nethaji *et.al.*, 1988).

FA, one of the most abundant phenolic acid in plant has received great attention in oriental research due to its ability as natural antioxidant. According to Soobrattee *et al* in 2005, like other well-known antioxidant such as Vitamin C and Vitamin E, natural antioxidant in plants phenolic also exhibit therapeutic potential for a variety of disease such as cancer, diabetes and cardiovascular diseases. Besides that, the restriction of using synthetic antioxidant such as BHA and BHT in food increases the interest of extracting natural antioxidants

FA is a ten carbon ($C_{10}H_{10}O_4$) organic acid compound that usually appears as powder at room temperature. Figure 2-1 shows the two isomeric forms of FA and Table 2-1 summarizes the physical and chemical properties of FA.

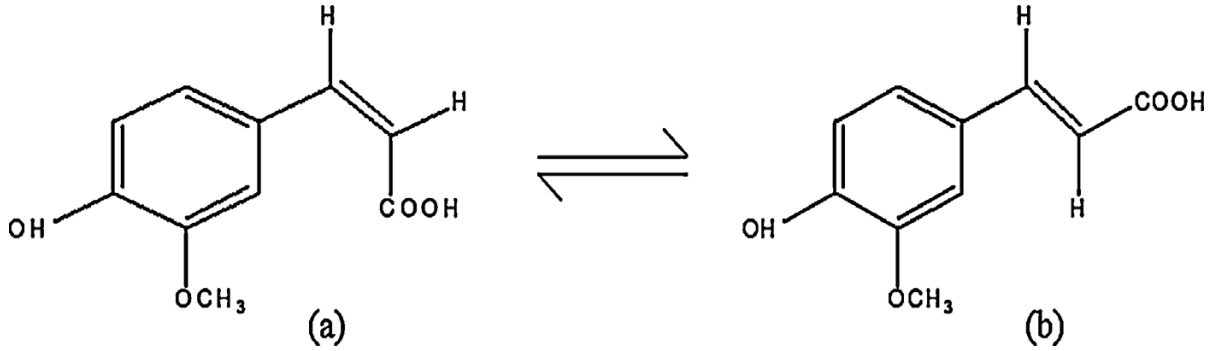

Figure 2.1: Schematic representation of two different isomeric forms of FA found in nature (a*) cis* conformation and (b) *trans* conformation of ferulic acid (Kumar & Pruthi, 2014)

Table 1: Physical and chemical properties of ferulic acid (Material Safety Data Sheet, www.mpbio.com)

Physical Properties	
Molecular Formula	$C_{10}H_{10}O_4$
Molecular Weight	194.18 g/mol
Physical State	Solid
Physical Appearance	Powder
Density	1.317 g/cm^3
Melting Point	168-172°C
Boiling Point	372.31°C
Chemical Properties	
Solubility	Soluble in hot water or ethyl acetate
Safety	Causes skin irritation, serious eye irritation and may cause respiratory irritation.
Flammability	Not flammable
Toxicity	Slightly hazardous for water

2.3 Application of Ferulic Acid

Ferulic acid has both industrial and biological usage. FA usage in biomedical includes FA that acts as antioxidant, antiallergic, hepatoprotective, anticarcinogenic, anti-inflammatory, anti-microbial, antiviral, vasodilatory effect and antithrombotic (Kumar *et al*., 2014).

Meanwhile industrial application of FA includes in food preservative industry; FA act as a cross linking agent (Oosterveld et al., 2010) and in cosmetic industry; FA act as photoprotective constituents in sunscreen and skin lotions (Saija *et al*., 2000). Table 2-2 below summarizes some of the industrial and biological usages of FA.

Table 2: Application of Ferulic Acid in Various Fields

Ferulic Acid Application	References
Antioxidant Ferulic acid acts as an antioxidant by scavenging free radicals and enhancing the cell stress response through the up-regulation of cytoprotective systems.	(Mancuso *et al*., 2013)
Anti-diabetic FA helps in neutralizing the free radicals present in the pancreas which is produced by the use of streptozotocin; a chemicals usually used in drugs for Type 1 Diabetes, thus it decreases the toxicity of streptozotocin. The reduce level of streptozotocin toxicity help ß-cells to secrete more insulin and decreases blood glucose level.	(Balasubashini *et al*., 2004)
Preclusion of Food Discoloration FA has been used to maintain the colour of green peas, prevent discoloration of green tea and oxidation of banana turning black colour by reducing bacterial contamination.	(Masaji, 1999)
Uses in Cosmetics FA possesses a structure similar to tyrosine and it inhibits the melanin formation through competitive inhibition with tyrosine, which gives considerable protection to the skin against UVB-induced erythema. FA is also a strong UV absorber.	(Saija *et al*., 2000)

2.4 Production of Ferulic Acid

Ferulic acid can be produced via two ways; by chemical synthesis and by bioprocess. Many enzymatic, acidic and alkaline methods have been proposed in literature in order to acquire FA.

2.4.1 Production via Chemical Synthesis

Production of FA via chemical synthesis is by condensation reaction of vanillin with malonic acid catalyzed by piperidine. This method produces FA as a mixture of *trans*- and *cis*-isomers. This method produces high yield, however it take several weeks to complete the reaction and produces FA (Ou *et al*., 2007).

2.4.2 Production via Bioprocess

Production of FA via bioprocess usually includes alkaline hydrolysis and enzymatic methods to release FA from natural sources. However, isolation of FA by enzymatic method is a difficult challenge because most of FA contents in plants are covalently linked with lignin and other biopolymers (Kumar & Pruthi, 2014). Although difficult, this method however received a large attempt because FA obtained from chemical synthesis cannot be considered natural. Since FA is a phenolic antioxidant ubiquitously present in plants, it would therefore be present in lignocellulosic biomass. Lignocellulosic biomass is referred as plant biomass such as corn fiber, corn cob and sugarcane bagasse. It composes of various compositions of cellulose, hemicellulose and lignin, depending on type of the lignocellulosic biomass (Hasyierah *et al*., 2008).

In 2008, Hasyierah *et al*. has written a journal on production of ferulic acid from lignocellulosic biomass by microbial transformation. A number of lignin, cellulose and hemicellulose can be released from lignocellulosic biomass after it undergoes pretreatment. Pretreatment of the lignocellulosic biomass includes chemical pretreatment, steam pretreatment and biological pretreatment. Biotransformation of phenolic compound like FA can then be carried out by various microorganisms such as lactic acid bacteria, yeast and fungal species.

In Malaysia, lignocellulosic biomass that is abundantly obtainable and cheap comes from the by-products of the palm oil industry. In 2011, Jamal *et al.* studied the production of phenolic acid from palm oil mill effluent under liquid-state fermentation by *A. niger*. Conventionally, FA is obtained by extraction and hydrolysis method. However, microbial fermentation through enzymatic hydrolysis offers an alternative to the conventional method. This method improves extraction yield, quality of the extracts and is also environmental friendly.

Based on all the encouraging evidence of production of FA by microbial fermentation of lignocellulosic biomass, it is come to intention to study the production of FA from fiber pressed oil palm frond, one of palm oil by-product, using mixed culture. Like other lignocellulosic biomass, OPF contain lignin, cellulose and hemicellulose (Zahari *et al*., 2012). Therefore based on this similarity, it is to be studied whether FPOPF could yield FA like the other lignocellulosic biomass or not.

2.4.3 Parameters Affecting Production via Bioprocess

In fermentation, the productivity of the microbial metabolite can be improved by manipulating its nutritional and physical parameters (Jamal *et al*., 2011). The physicochemical parameters such as carbon and nutrients composition, incubation temperature, pH, inoculum size and agitation rate have been observed to influence the microbial growth and the production of desired products (Ellaiah *et al*, 2004). Since no study has been carried out on production of FA from FPOPF using mixed culture, it is assumed that the general parameters affecting other microbial growth in fermentation will affect microbial production of FA from FPOPF.

2.5 Fiber Pressed Oil Palm Frond

OPF is abundantly obtainable in Malaysia, due to the fact that Malaysia is the second world's leading palm oil producing country after Indonesia. Palm oil wastes such as OPF, oil palm trunk (OPT) and oil palm empty fruit brunch (OPEFB) are generated and discarded daily as agricultural wastes by the palm oil industry. Fiber pressed oil palm frond (FPOPF) is obtained by simply grinding the oil palm frond into fiber form. Figure 2-2 shows the FPOPF that is obtained after grinding the oil palm frond.

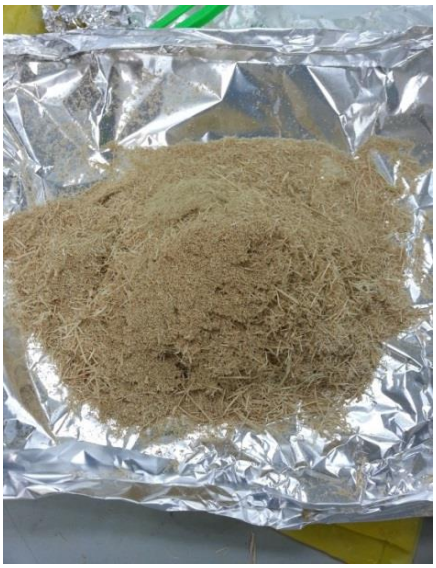

Figure 2.2: Fiber Pressed Oil Palm Frond

For the past years, OPEFB and OPT have been the common palm oil waste used as substrate for fermentable sugar production (Zahari *et al*., 2012). However these two palm oil wastes have disadvantages, such as difficulties to be hydrolyzed and not as abundant as OPF. It has been reported that other than containing low metal contents, OPF has high carbohydrates in the form of simple sugars (Zahari *et al*., 2012). Therefore according part of the OPF can be utilized for other purposes without disturbing the nutrient recycling process.

2.6 Mixed Culture Fermentation

Mixed culture fermentation is a biochemical process involving two or more microorganisms in a nutrient substance in specially controlled conditions for scientific, medical or commercial purposes (Opara *et al*., 2013). Mixed culture composition varies depending on the growth condition needed. As stated by Lin *et al*. in 2011, original mixed cultures are structured by highly diverse microbes and constructed from natural inoculum. This condition facilitates biological processes under non-sterile conditions without altering strain stability. In this study, it is intended to use a mixed culture of *A.niger, Streptomyces* sp. and *Penicillium* sp.

3 MATERIALS AND METHODS

3.1 Overview

During a fermentation process, the basic steps included are substrate preparation, inoculum preparation, experimental setup and the analysis of sample. This chapter describes in details all the mentioned steps during the production of FA from FPOPF using mixed culture.

3.2 Raw Materials and Chemicals

The OPF was obtained from one of the palm oil plantation located at Lepar Hilir. These OPF were squeezed and pressed first for their juice and sap, however only the fiber from the pressing process was used for this study. The fiber-pressed oil palm frond (FPOPF) were kept in freezer for storage purposes.

As for the preparation of standard calibration curve, 99% FA was purchased from Sigma Aldrich (Malaysia). For the mobile phase in HPLC (high performance liquid chromatography) analysis, the Acetonitrile HPLC grade purchased from Fisher Scientific (Malaysia) was used.

3.3 Inoculum Preparation

The acclimatized FPOPF soil culture was used as the inoculum in this experiment. First of all, the slurry mixture of FPOPF and water were frozen in small packets of 60mL volume. These packets were ready to be thawed and poured into the bioreactor. The calculation of FPOPF and soil used can be referred as below.

Calculation of FPOPF and soil used:-

$$FPOPF\ juice\ per\ day = \frac{Working\ volume}{30\ days}$$

$$Working\ volume = 3600\ mL$$

Hence;

$$FPOPF\ juice\ per\ day = 120\ mL$$

a. It is discovered that at 1:10 ratio of FPOPF weight to water volume, FPOPF mixture is in slurry condition.

b. 360 g FPOPF and 3600 mL water were mixed together. The mixture was stirred to achieve well mix condition.

c. The mixture was poured into small seal plastic bags of 60 mL volume. Two of these packets will be poured into the bioreactor on a daily basis for 30 days.

Then the acclimatization was done by mixing the soil of the oil palm tree with the small packets of FPOPF in a reactor. The acclimatization took 30 days at ambient temperature before it is ready to be used as inoculum (Kushairi & Zainol, 2011). Detailed preparation of the inoculum can be referred to Appendix A.

3.4 Substrate Preparation

As for the substrate, a mixture of FPOPF and distilled water at the ratio of 1:10 was used. 10 g of FPOPF is added with 100 mL of distilled water. The FPOPF and distilled water were mixed during experimental setup (Kushairi & Zainol, 2011).

3.5 Experimental Setup

The parameters that will be manipulated in this experiment were determined. Those parameters were pH value (pH 5 to pH 9), temperature (26°C to 40°C), fermentation time (1 to 3 days), agitation (with 150 rpm agitation and without agitation) and substrate-to-inoculum ratio (2%-10%). After the identification of the parameters, all the parameters were tabulated into standard runs using Design Expert software.

The inoculum and substrate were mixed together in a 250 mL conical flask. The ratio of the inoculum to substrate, temperature, agitation, pH value and other factors were all based on the standard runs exhibit by the Design Expert software as shown by table 3.1. The conical flasks were then incubated in the incubator shaker (Jamal *et al.*, 2011).

Table 3: Experimental Design Layout using 25 Fractional Factorial Design with Response

Run	Temperature (°C)	pH	Agitation (rpm)	Time (day)	Inoculum Percentage %
1	26	9	0	3	10
2	40	5	0	1	2
3	26	5	150	3	10
4	26	5	0	1	10
5	40	9	150	1	2
6	26	5	0	3	2
7	40	9	0	3	2
8	26	9	0	1	2
9	40	5	150	1	10
10	40	5	0	3	10
11	26	5	150	1	2
12	40	9	150	3	10
13	26	9	150	1	10
14	26	9	150	3	2
15	40	9	0	1	10
16	40	5	150	3	2

3.6 HPLC Analysis

The detection for the presence of FA was done for the fermented liquid by using a Waters (Milford, MA, USA) HPLC chromatography. The HPLC chomatograph was equipped with a 600-MS controller, a 717 plus autosampler and a 996 photodiode-array detector. A gradient of solvent A (water–acetic acid, 98:2, v/v) and solvent B (water–acetonitrile–acetic acid, 78:20:2, v/v/v) applied to a reversed-phase Nova-pack C18 column (30 cm × 3.9mm i.d.) as follows: 0–55 min, 80% B linear, 1.1 ml/min; 55–57 min, 90% B linear, 1.2 ml/min; 57–70 min, 90% B isocratic, 1.2 ml/min; 70–80 min, 95% B linear, 1.2 ml/min; 80–90 min, 100% B linear, 1.2 ml/min; 90–120 min, washing and re-equilibration of the column (Hernanz *et al.*, 2001).

Before running the detection for the sample, detection was done for pure FA solution to obtain the standard chromatogram for FA. Then detection was performed by scanning the sample at 280nm. Identification of chromatographic peaks was carried out by comparing the chromatogram obtained to those of standards. Quantification of total FA is carried out by area measurements at 280 nm (Jamal *et al.*, 2011).

4 RESULTS AND DISCUSSION

4.1 Overview

The results of FA yield obtained were tabulated into the Design Expert software and analysis of variance (ANOVA) was performed to determine which independent factors gives the highest effect to this research. The discussion on the result obtained covers the effect list, the Half-Normal Plot, the Pareto Chart and the ANOVA table.

4.2 Fractional Factorial Screening

The yields of FA produced by each runs were determined using HPLC. Table 4.1 below shows the yield of mg of FA produces for every kg of FPOPF used.

Table 4: Experimental Design Layout using 2^5 Fractional Factorial Design with Response

Run	Temperature (°C)	pH	Agitation (rpm)	Time (day)	Inoculum Percentage %	Yield (mg FA/kg FPOPF)
1	26	9	0	3	10	2.6318
2	40	5	0	1	2	3.7239
3	26	5	150	3	10	29.8795
4	26	5	0	1	10	9.2339
5	40	9	150	1	2	26.7578
6	26	5	0	3	2	3.3616
7	40	9	0	3	2	3.5444
8	26	9	0	1	2	101.5940
9	40	5	150	1	10	4.2365
10	40	5	0	3	10	3.9680
11	26	5	150	1	2	4.4138
12	40	9	150	3	10	6.3509
13	26	9	150	1	10	89.7187
14	26	9	150	3	2	129.7895
15	40	9	0	1	10	11.6328
16	40	5	150	3	2	1.9695

4.3 Main Effect Analysis

From Table 4.2 it can be seen that pH value gave the highest contribution percentage which was 23.76%. The standardized effect of pH was 38.90, which means the maximum pH value (pH 9) gives higher contribution compared to the minimum pH value (pH 5). According to Friedman & Jurgens (2000), the phenolic compound in lignocellulosic biomass contains a few different types of acid for example caffeic acid,

cinnamic acid, cholorogenic acid and ferulic acid. All this acid has their own range of pH in which they will be in a stable condition. The range of pH used will also be one of the factors that will determine the type of acid release from the phenolic compound. In previous research by Friedman & Jurgens on the effect on pH on the stability of plant phenolic compounds, it is found that FA is more stable at high pH (pH 7-9). Caffeic and cholorogenic acid however, are not stable at high pH. At different pH, different acid will be released from the phenolic compound therefore making pH a major factor contributing to the FA production from the FPOPF.

The second highest factor contributing to the FA production was temperature, which contributes 23.34%. The standardized effect of temperature was -38.55, showing that the minimum temperature of 26°C gives higher contribution compared to higher temperature of 40°C. According to Xie *et al.* (2010), the secretion of cellulase and ferulic acid esterase (FAE) allowed the release of ferulic acid in wheat bran (wheat bran is similar to lignocellulosic biomass, however it does not contain lignin). Szwajgier *et al.* (2006) stated that the FAE is not thermostable and a decrease in FAE activity was observed during mashing when the medium temperature was at 35°C. Another research by Tilash *et al*. in 2008 on the preparation of FA from agricultural waste stated that, the optimum condition to obtain FA from extraction was at medium temperature of 21.6°C. All these previous researches conclude that too high of a medium temperature can inhibit the FAE activity therefore preventing the release of FA. This shows that temperature is one of the important factors in FA production.

Another factor that was taken into account during the production of FA from FPOPF was agitation. The standardized effect of this factor is 19.18, which indicates that the higher agitation (150 rpm) gives more contribution to ferulic acid yield compared to 0 rpm agitation. Instead of being in a liqudised condition, the substrate were in a slurry condition, thus agitation helps the inoculum to be distributed thoroughly to the substrate's surface area during the fermentation. However the effect of this factor was not so significant as it only gave the percentage contribution of 5.77%.

The inoculum percentage or the substrate to inoculum ratio contributes as much as 3.39%. Based on the standardized effect of -14.69, it can be seen that the lower percentage of inoculum (2%) was better than the higher percentage (10%). This shows

that there was a limit amount of mixed culture needed to release FA from the FPOPF. Higher presence of mixed culture does not gives higher FA yield and higher amount of FPOPF (more than 98%) might be too handful for the mixed culture to breakdown.

The least contributing factor was the fermentation time, which only contributes as much as 1.20%. In many previous research of FA release from agricultural waste , it was found that the typical optimum fermentation time was 24 hour. (Huang *et al*., 2011) (Jamal *et al*., 2011). Since this factor was the least affecting factor in this research, the value should be fixed for further optimization research.

Table 5: The Percentage Contribution of Each Factor and Their Interaction in FA production from FPOPF

Term	Standardized Effects	Sum of Squares	% Contribution
A-Temperature	-38.55	5945.91	23.34
B- pH	38.90	6054.13	23.76
C- Agitation	19.18	1471.22	5.77
D- Time	-8.73	304.64	1.20
E- Percentage Inoculum	-14.69	862.93	3.39
AB	-30.31	3674.09	14.42
AC	-15.07	908.04	3.56
AD	1.10	4.82	0.019
AE	12.24	598.87	2.35
BC	14.13	798.09	3.13
BD	-13.12	688.50	2.70
BE	-23.15	2143.70	8.41
CD	19.44	1512.07	5.93
CE	6.50	169.08	0.66
DE	-9.27	343.80	1.35

4.4 Half-Normal Plot

Half-normal plot shows the magnitude of the experiment's effects as "Standardized Effects", ordered in increasing magnitude, along the x-axis. This plot also shows which factors have significant effect to the responses and which is not. The further the point from the red line, the more significant effect it has on the corresponding response (Woolf, 2006). The color of the data points indicates whether the original effect is positive (yellow) or negative (blue). As shown in Figure 4.3, factors that were manually selected by author are represented by the half-coloured point; meanwhile factors that were not selected are represented by the fully-coloured point.

The factors manually selected by author were temperature (A), pH value (B), agitation (C), fermentation time (D), inoculum percentage (E), interaction between temperature & pH value (AB), interaction between pH value & inoculum percentage (BE), interaction between agitation rate & fermentation time (CD) and interaction between temperature & agitation rate (AC). The furthest point from the red line was the pH, indicating that pH value gives the most significant effect to the response, followed by temperature as second highest significant factor. The interaction between temperature & pH value (AB) can be considered significant since the point AB also falls off the straight line, same as point A and point B.

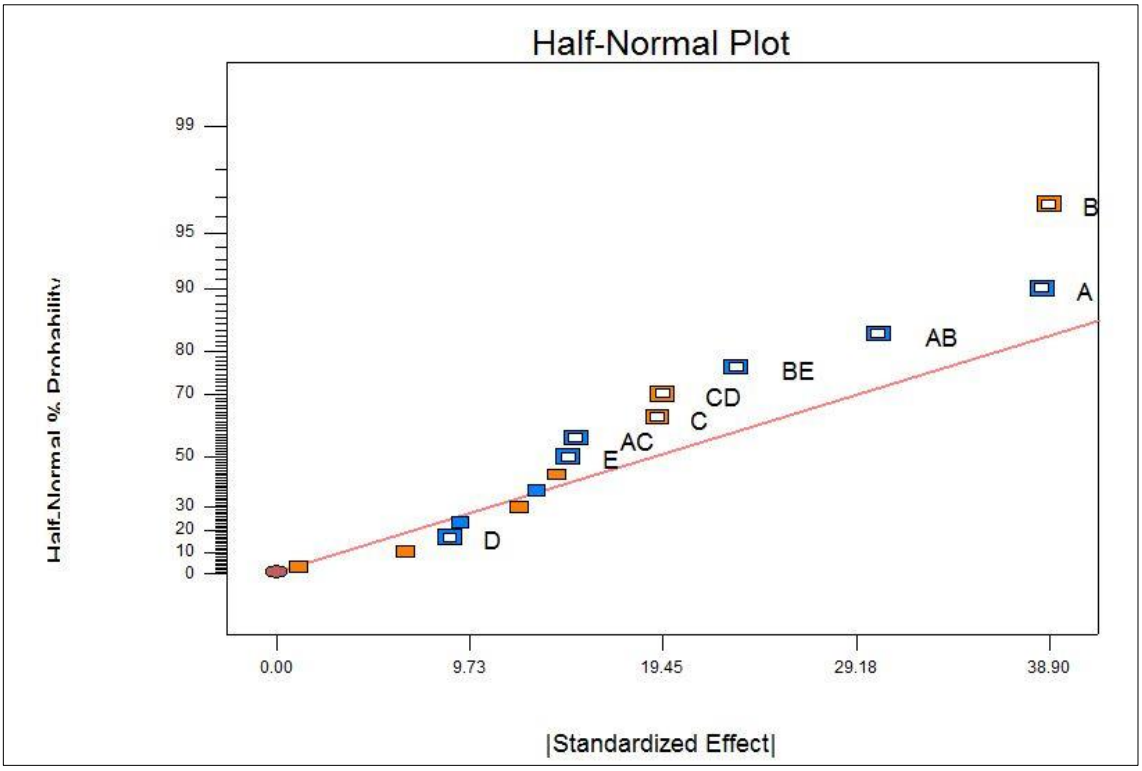

Figure 4.1: The Half-Normal Plot of Standardized Effect vs Half-Normal Probability; A: Temperature, B: pH Value, C: Agitation, D: Fermentation Time, E: Inoculum Percentage; Half-coloured points represent the manually selected factors; Fully-coloured points represents unselected factors.